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Mitogen-Induced Genes Are Subject to Multiple Pathways of Regulation in the Initial Stages of T-Cell Activation

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The delivery of a mitogenic signal to T cells via any one of several cell surface molecules elicits a variety of intracellular responses, some or all of which regulate subsequent gene expression events. The expression of nine novel mitogen-induced genes in response to various T-cell-activating agents was examined to evaluate the diversity of pathways which regulate such genes. The relative contribution of distinct secondary signals, individually or together, to mitogen-stimulated gene induction and the capability of individual genes to respond to the sometimes divergent signals generated from different cell surface structures is addressed. The activation of T cells with mitogenic monoclonal antibodies directed against the CD2 or CD3 cell surface molecules, or with phytohemagglutinin, induced all nine genes. Thus, stimulation by fully mitogenic agents regardless of cell surface-binding specificity correlated with the expression of all of the genes studied. However, heterogeneous patterns of gene expression, encompassing five regulatory classes, were revealed by the use of phorbol 12-myristate 13-acetate, calcium ionophore, and anti-CD28 monoclonal antibody, agents which mediated only a subset of intracellular events and thus an incomplete mitogenic signal. Interleukin-2 and two novel lymphokines represented one regulatory class that appeared to require unique transcriptional activation signals relative to the other mitogen-induced genes. As demonstrated in the accompanying paper (P. F. Zipfel, S. G. Irving, K. Kelly, and U. Siebenlist, Mol. Cell. Biol. 9:1041–1048, 1989), the immediate transcriptional response of T cells to mitogenic stimulation is quite complex, involving numerous genes beyond those which have been previously described. Furthermore, the discrimination of several regulatory phenotypes among these nine genes suggests that a multiplicity of signaling pathways extends from the cell surface to the level of transcription.

In the accompanying paper (27), we describe the isolation of more than 60 distinct, novel cDNA clones derived from immediate early mRNA species that are induced after the mitogenic activation of quiescent human peripheral blood (PB) T cells by phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA). Our data suggest that the initial biochemical response to a stimulus delivered to T cells is highly complex, involving a large number of directly induced genes. It is expected that distinct pathways mediated by characteristic secondary signals induce groups of genes within this large family. Indeed, an important question, which is addressed in this report, concerns the homogeneity or heterogeneity of induced gene responses elicited by those stimuli which lead to proliferation. Genes that play an essential role in cell cycle progression are expected to be uniformly expressed during any mitogenic response, regardless of initiating agent. In addition, it is important to define potential regulatory classes of induced genes in order to ultimately elucidate regulatory motifs characteristic for a given class.

In this study, we have characterized in detail the activation requirements of nine novel mitogen-induced genes by using various mitogenic and comitogenic agents that are known to effect distinct secondary signals (summarized in Fig. 1). These nine genes were selected for further study from the larger group described in the accompanying paper (27) because their expression was limited to lymphoid cells or because they displayed interesting regulatory properties such as inhibition of induction by the immunosuppressive drug cyclosporin A (27) or elevated expression in response to human T-cell leukemia virus type I infection. The activating agents examined in this study included monoclonal antibodies directed against CD2, CD3, or CD28 which are distinct, physically nonassociated T-cell surface molecules, the lectin PHA, calcium ionophore, PMA, and dioctanoylglycerol (DiC8).

The binding of antigen or monoclonal antibody to the T-cell receptor or the associated CD3 complex of T cells initiates the activation of T cells (2, 18, 23). PHA-mediated stimulation appears to require the presence of the antigen receptor complex or a coordinately expressed molecule, as determined by studies with selective surface loss mutants (20). An antigen-independent pathway of T-cell activation involving the CD2 complex (E-rosette receptor) has also been described (19). Binding of these mitogens to cell surface receptors on T cells results in the production of inositol 1,4,5-triphosphate, an increased intracellular calcium concentration ($[Ca^{2+}]_i$), membrane translocation of protein kinase C (PKC), and subsequent proliferation (6, 9, 13, 15, 22, 26). In contrast, the signal delivered by CD28 seems to be fundamentally distinct from that of CD2 or CD3 because stimulation by anti-CD28 leads to an elevation of cytoplasmic cyclic GMP concentration and does not cause increases in $[Ca^{2+}]_i$ or activation of PKC (12, 14, 22, 25). Furthermore, the interleukin-2 (IL-2) production initiated by CD28 is resistant to the effects of cyclosporin A, in contrast to that induced by CD3 or calcium ionophore and PMA (8). In addition, anti-CD28 alone does not stimulate proliferation of T cells but requires the simultaneous presence of a comitogen such as PMA (7, 8). T cells may be activated,

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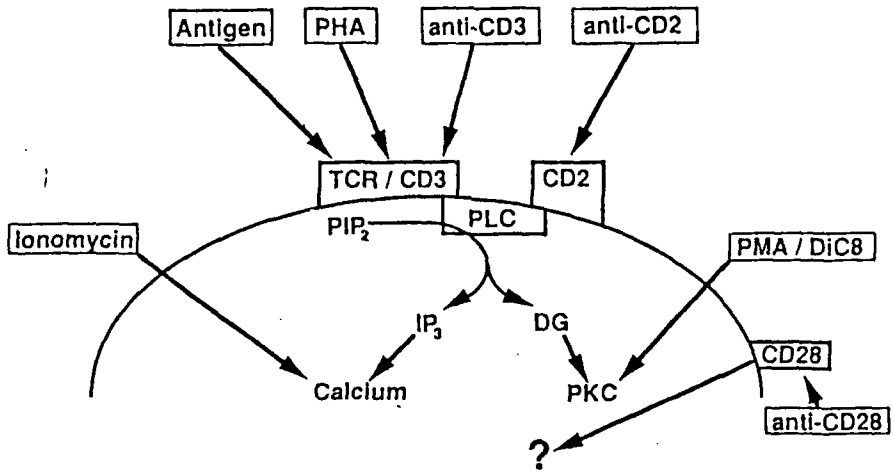

FIG. 1. Known intracellular signals generated by the stimulatory agents used. PLC, Phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate; DG, 1,2-diacylglycerol.

bypassing cell surface interactions, after treatment with calcium ionophore in addition to PMA or DiC8; the latter two agents act, at least in part, by stimulating PKC (16, 21). Ionophore-mediated increases in $[Ca^{2+}]_i$ act synergistically with DiC8 or PMA to stimulate proliferation and induced gene expression in purified PB T cells and induced expression of some genes, including lymphokine genes, in the T-cell line Jurkat (16; S. G. Irving, unpublished observations).

MATERIALS AND METHODS

Purification, culture, and activation of PB T cells. PB mononuclear cells were obtained from normal healthy donors by leukophoresis, and PB lymphocytes were isolated by density gradient centrifugation through lymphocyte separation medium (Litton Bionetics, Kensington, Md.), followed by removal of adherent cells (monocytes and B cells) by passage over a nylon wool column. The resulting cells had approximately 1% B cells and <1% monocytes as determined by flow cytometry. The T cells were cultured at 2×10^6/ml in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum and stimulated with one of the following agents: anti-CD3 (mAb OKT3; Ortho Diagnostics, Inc., Raritan, N.J.), used at 10 ng/ml; PHA-P (Burroughs Wellcome Co., Research Triangle Park, N.C.), used at 3 μg/ml; and anti-CD2 (19; anti-$T11_2$ and anti-$T11_3$ ascites), each ascites used at a 1:200 dilution. All reagents were titrated to determine the optimal concentration as assayed by ability to stimulate peak proliferation of PB T cells or IL-2 production by Jurkat cells. When included, cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was used at 10 μg/ml. The ability of each agent to stimulate proliferation was determined by [^{3}H]thymidine incorporation assayed after 4 days. A representative experiment showed 1,003 cpm for culture medium alone, 184,567 cpm for PHA, 107,908 cpm for anti-CD2, and 1,248 cpm for anti-CD3. The lack of response to soluble anti-CD3 in PB T cells appeared to be due to monocyte depletion, since unfractionated PB lymphocytes from the same donor gave 127,760 cpm.

Culture and activation of $CD28^+$ PB T cells. Approximately 80% of PB T cells express CD28; therefore, to ensure a maximal response by responding $CD28^+$ cells and to eliminate the potential interaction of $CD28^-$ and $CD28^+$ populations, $CD28^+$ cells were purified as previously described (8). The cells were cultured as described above and stimulated for 4 h with anti-CD28 monoclonal antibody 9.3 at 100 ng/ml, ionomycin (Calbiochem-Behring, La Jolla, Calif.; dissolved in dimethyl sulfoxide) at 133 nM, and PMA (Sigma; dissolved in dimethyl sulfoxide) at 0.3 ng/ml. A preliminary experiment showed all of the genes to be maximally induced at 4 h. The PMA concentration was determined by [^{3}H]thymidine incorporation to maximally synergize with either anti-CD28 or ionomycin yet not to be itself mitogenic. The mitogenic activity of each agent was assayed by [^{3}H]thymidine incorporation after 3 days of stimulation. A representative experiment showed 121 cpm for medium alone, 488 cpm for PMA, 236 cpm for anti-CD28 alone, 58,590 cpm for anti-CD28 and PMA, 186 cpm for ionomycin alone, 44,760 cpm for ionomycin and PMA, and 69,360 cpm for immobilized anti-CD3.

Culture and activation of Jurkat cells. Jurkat cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum at a density of 2×10^5 to 8×10^5/ml. Fresh portions of cells were thawed at approximately 6-week intervals. Cells were stimulated at 4×10^5/ml with one or more of the following agents: DiC8 (Molecular Probes, Inc., Eugene, Ore; dissolved in absolute ethanol) at 100 μM, ionomycin at 500 nM, and cycloheximide at 10 μg/ml. The DiC8 and ionomycin concentrations were determined by IL-2 production, assayed on the IL-2-dependent T-cell line CTLL, to maximally synergize with each other, although neither used alone resulted in any IL-2 production.

Northern (RNA) blot analysis. After culture for the indicated time points, total cellular RNA was extracted with guanidine thiocyanate and purified by centrifugation through a cushion of 5.7 M CsCl (3). RNA (10 μg per lane) was separated by electrophoresis in formaldehyde-agarose gels, blotted onto GeneScreen membrane filters (Dupont, NEN Research Products, Boston, Mass.), and hybridized to ^{32}P-labeled purified cDNA inserts prepared by nick translation.

RESULTS

In initial experiments, the expression of nine novel mitogen-induced genes was examined in PB nylon-wool-nonadherent T cells. The cells were stimulated for various amounts of time with (i) OKT3, a mitogenic monoclonal antibody that

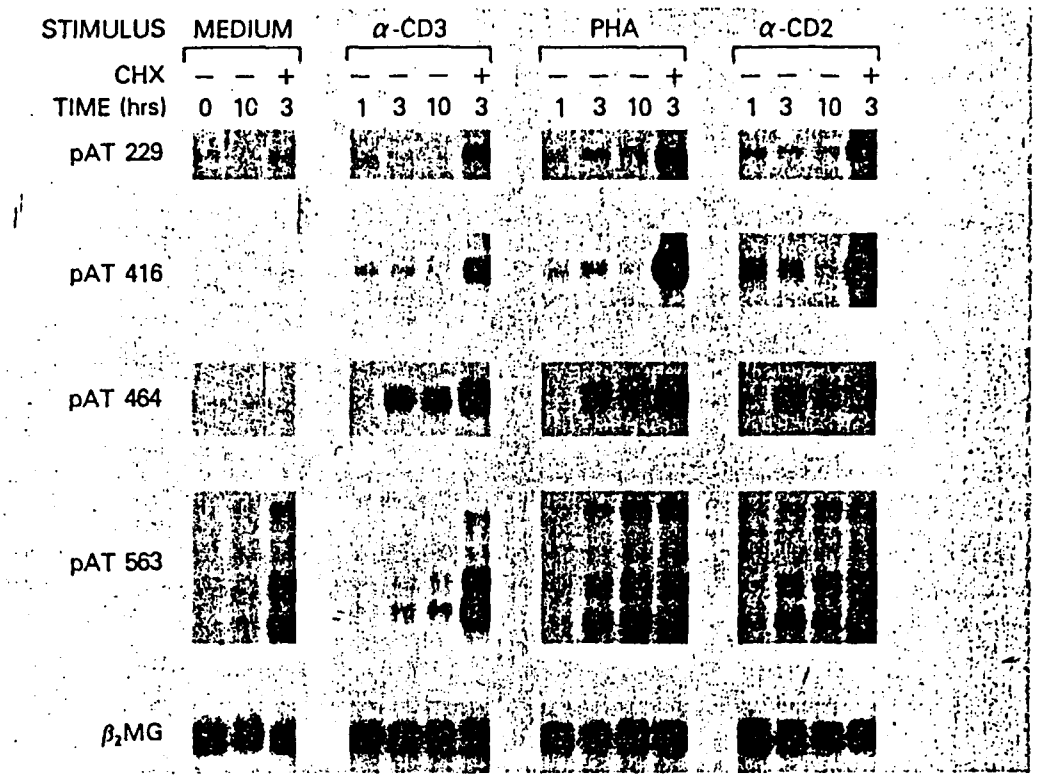

FIG. 2. Northern blot analysis of mitogen-induced genes in PB T cells. PB T cells were stimulated with either anti-CD3, PHA, or anti-CD2 in the absence or presence of cycloheximide (CHX) for the times indicated, and total cellular RNA was analyzed as described in Materials and Methods.

binds to a nonpolymorphic chain of the CD3 complex (1); (ii) a mitogenic combination of two monoclonal antibodies directed against the CD2 molecule (19); and (iii) PHA. Steady-state mRNA levels of the nine genes were determined.

For each gene, PHA, anti-CD2, and anti-CD3 stimulation resulted in similar responses. A hierarchy in which the relative levels of mRNA expression could be graded from a low level of inducibility (pAT 229) to strong inducibility (pAT 464) was seen in response to any of the three mitogens. Of the nine genes examined, two (pAT 464 [Fig. 2] and pAT 744 [data not shown]) were strongly induced, two (pAT 563 [Fig. 2] and pAT 120 [data not shown]) were induced to a moderate level, two (pAT 416 [Fig. 2] and pAT 237 [data not shown] were induced to a low level, and three (pAT 229 [Fig. 2] and pAT 154 and pAT 225 [data not shown]) were weakly induced either by PHA or by anti-CD2 or anti-CD3. For all of the genes, stimulation with any one of the above mitogens in the presence of PMA elicited a greater response than did stimulation with PHA or with anti-CD2 or anti-CD3 alone (data not shown). In addition, the kinetics of mRNA accumulation, although unique for each gene, were relatively uniform for each gene irrespective of the stimulus used.

Cells were stimulated in the absence or presence of cycloheximide to determine whether the interruption of protein synthesis would affect gene expression; previous studies have shown that the mRNA levels of many induced genes are elevated under these conditions (4, 10, 11). The relative effects of addition of cycloheximide together with the mitogenic agent were consistent among the various stimuli for each gene examined, even though the magnitude of the cycloheximide-related modulation of mRNA levels differed among the genes from virtually undetectable to greater than 10-fold.

These results (summarized in Table 1) show that these novel genes are induced by mitogenic agents other than those used to prepare RNA for the library (27). Importantly, stimulation via monoclonal antibody binding to one chain within the antigen-binding receptor (CD3) induced expression of the novel genes studied here. Furthermore, we have observed similar gene induction in human T-helper clones after addition of appropriate antigen and antigen-presenting cells. Thus, when T cells were activated through either the CD2 or CD3 cell surface receptors or with PHA, each gene in this panel of induced genes demonstrated a consistent kinetic pattern and level of expression irrespective of the agent used to generate these signals.

Despite the binding to different cell surface receptors, each of the above mitogens has been shown to generate similar immediate intracellular responses, part or all of which may be required for activation of the nine genes examined in Fig. 2. Since the conditions used in this experiment did not result in proliferation for one of the three stimuli used (anti-CD3; see Materials and Methods), expression of these nine genes was not sufficient to initiate DNA synthesis.

To further examine whether the induction requirements of the nine genes could be grouped into families, highly purified T cells were stimulated with comitogenic agents that generate distinct biochemical signals. These agents included calcium ionophore, PKC activators, and anti-CD28, none of which stimulates DNA synthesis in purified T cells (8, 16, 24; reviewed in reference 25; C. H. June, J. A. Ledbetter, T.

TABLE 1. Summary of Northern blot data for mitogen-induced genes in response to agents used in Fig. 1 through 3[a]

Class		Total PB T cells: anti-CD3, anti-CD2, and PHA induction	CD28+ PB T cells				Jurkat cells			
			PMA induction	Ionomycin induction	Ionomycin and PMA synergy	Anti-CD28 and PMA synergy	DiC8 induction	Ionomycin induction	Ionomycin and DiC8 synergy	Effect of cycloheximide on expression
I.	pAT 464	+	±	−	S	S	−	−	S	Ab
	pAT 744	+	−	−	S	S	−	−	S	Ab
	IL-2	+	−	−	S	S	−	−	S	Ab
II.	pAT 237	+	+	−	S	S	+	−	NS	Incr
III.	pAT 563	+	+	−	NS	S	ND	ND	ND	ND
IV.	pAT 229	+	+	−	NS	NS	+	+	S	Incr
V.	pAT 120	+	+	−	NS	NS	+	−	NS	Incr
	pAT 154	+	+	−	NS	NS	+	−	NS	Incr
	pAT 225	+	+	−	NS	NS	+	−	NS	Incr
	pAT 416	+	+	−	NS	NS	+	−	NS	Incr
VI.	IL-2R	+	+	−	S	S	+	−	S	ND

[a] Abbreviations: +, mRNA hybridizing to labeled probe detected; −, no hybridization; ±, weak hybridization; S, synergistic effect on mRNA levels; NS, no synergistic effect noted; Ab, abrogation of DiC8- and ionomycin-mediated expression; Incr, increased levels of DiC8- and ionomycin-mediated expression; ND, not done.

Lindsten, and C. B. Thompson, J. Immunol., in press). In contrast, purified T cells proliferated after treatment with the combination of calcium ionophore or anti-CD28 and PMA (see Materials and Methods).

Steady-state mRNA levels resulting from stimulation of CD28+ T cells with PMA, anti-CD28, ionomycin, PMA plus anti-CD28, and PMA plus ionomycin are shown in Fig. 3. Unlike stimulation via CD2 or CD3, which elicited graded responses from all nine mitogen-induced genes, activation through CD28 clearly divided the nine novel genes into distinct groups (Fig. 3 and Table 1). One group, exemplified by pAT 416 (Fig. 3) and including pAT 120, pAT 154, pAT 225, and pAT 229 (data not shown), showed no response to anti-CD28. This group of genes displayed variable responsiveness to PMA alone, but in no case was potentiation of the PMA signal by anti-CD28 observed. Stimulation of cells with control monoclonal antibodies binding to CD4, CD8, or CD45 did not affect steady-state mRNA levels. However, steady-state mRNA levels observed with PMA treatment alone were elevated by costimulation with PHA and PMA (data not shown). In the second class of genes, anti-CD28 responsiveness was observed as a synergism between anti-CD28 and PMA for four novel genes (pAT 237 and pAT 464 [Fig. 3] and pAT 563 and pAT 744 [data not shown]) and for IL-2R (8; data not shown). Although PMA alone induced various levels of expression, gene induction with soluble or cross-linked anti-CD28 alone was not seen in this group. IL-2 mRNA, in contrast, was not induced by PMA alone but was induced in response to a synergistic combination of PMA and anti-CD28. All nine mitogen-induced genes responded to immobilized anti-CD3 (data not shown), which demonstrated that the gene expression patterns in the CD28+ PB T cells were comparable to those of the less rigorously purified PB T cells used in Fig. 2.

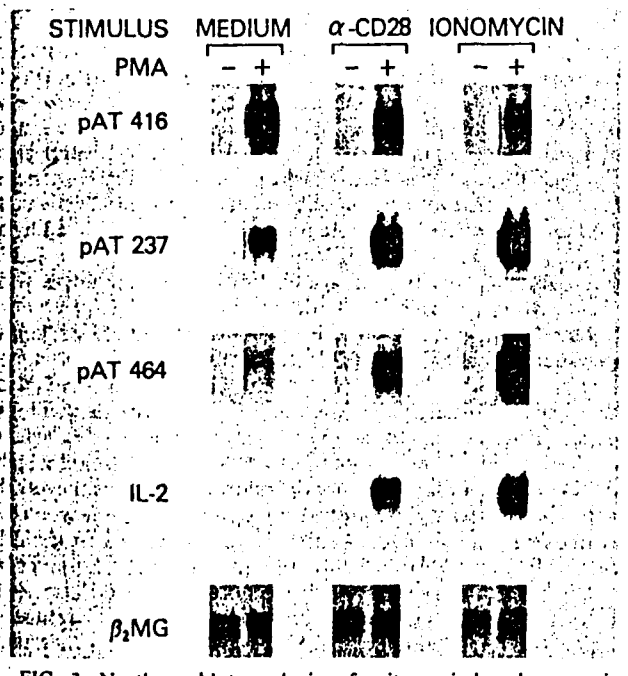

FIG. 3. Northern blot analysis of mitogen-induced genes in CD28+ T cells. CD28+ T cells were stimulated for 4 h with either anti-CD28 or ionomycin in the absence or presence of PMA, and total cellular RNA was analyzed as described in Materials and Methods.

These results extend the data presented in Fig. 2 and demonstrate that by using an alternative means of activating PB T cells, nine mitogen-induced genes which all responded to PHA-, anti-CD2-, or anti-CD3-mediated signals could be clearly divided into a group that responded to CD28-derived signals (in the presence of PMA) and a group that did not respond to such signals (Table 1). For those genes that responded to anti-CD28 treatment, either signals common to CD28-, CD3-, and CD2-mediated activation may regulate some of these genes or anti-CD28 delivers a novel signal that

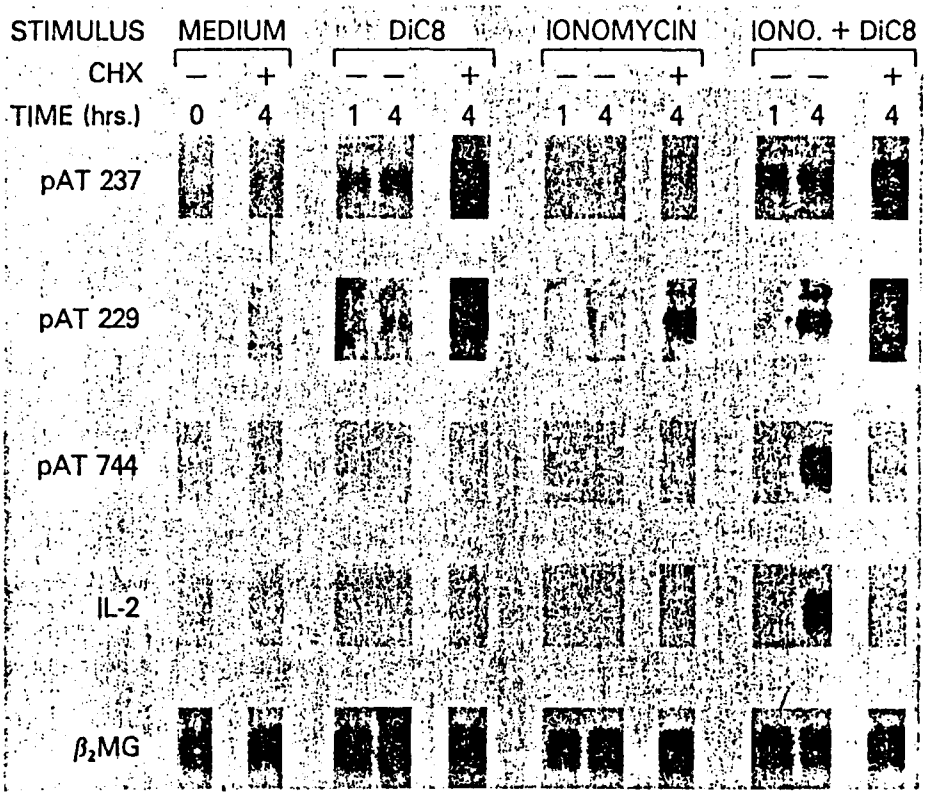

FIG. 4. Northern blot analysis of mitogen-induced genes in the T-cell line Jurkat. Jurkat cells were stimulated with either DiC8 or ionomycin or both in the absence or presence of cycloheximide (CHX) for the times indicated, and total cellular RNA was analyzed as described in Materials and Methods.

can induce expression of some genes by an alternative mechanism.

To compare the effects of the comitogens PMA and anti-CD28 with those of another comitogenic agent, Ca^{2+} ionophore, we treated the cells with ionomycin in the absence or presence of PMA. Interestingly, three of four of the genes for which synergy between anti-CD28 and PMA was observed (pAT 237 and pAT 464 [Fig. 3] and pAT 744 [data not shown]) displayed strong synergy between ionomycin and PMA, and anti-CD28-nonresponsive genes were likewise unaffected by ionomycin. Anti-CD28 does not appear to affect $[Ca^{2+}]_i$ levels (12, 14), and the effects of ionomycin are attributable to elevated $[Ca^{2+}]_i$ (24). This result, together with the observation that pAT 563 was found to be regulated differently by anti-CD28 and ionomycin in the presence of PMA (Table 1), suggests that the mechanisms by which these two partially mitogenic agents synergize with PMA are likely to be different.

To further define regulatory families of mitogen-induced genes, we examined the separate and combined effects if ionomycin- and DiC8-mediated PKC activation on gene induction in the T-cell line Jurkat. DiC8 is a cell-permeant synthetic diacylglycerol that mimics the effect of endogenous diacylglycerol by reversibly activating PKC (5, 16, 17). Our previous studies have shown that eight of the nine mitogen-induced genes analyzed as described above in resting T cells were inducible in the Jurkat line with the combined addition of PHA and PMA; pAT 563 was constitutively expressed at a low level (data not shown). Treatment of Jurkat cells with ionomycin, DiC8, or both in the presence or absence of cycloheximide showed that three regulatory classes of genes could be defined. The first class (represented by pAT 237 in Fig. 4) included genes that were PKC inducible but displayed either variable and minimal or no synergy with a $[Ca^{2+}]_i$ signal (pAT 237 [Fig. 4] and pAT 120, pAT 154, pAT 225, and pAT 416 [data not shown]). No response to ionomycin alone was detected for this class of genes. All of these genes were similarly induced in PB T cells by PMA-mediated PKC activation (Fig. 3 and data not shown), and the patterns of gene expression in response to PMA in Jurkat cells were identical to those presented here for DiC8 (data not shown). A second class, consisting of pAT 229, was PKC inducible and able to be potentiated by a $[Ca^{2+}]_i$ signal. In this regard, pAT 229 resembled IL-2R (data not shown) but differed from IL-2R in that it was induced by increased $[Ca^{2+}]_i$ levels alone (Fig. 4).

The final class observed in these cells included pAT 464 (data not shown) and pAT 744 as well as IL-2 (Fig. 4), whose induction exhibited a requirement for both signals assayed in this experiment. In addition, the responses of the various genes in Jurkat cells to the inducing agents in the presence of cycloheximide demonstrated an additional correlation which distinguished IL-2, pAT 464, and pAT 744 from the other genes. Specifically, cycloheximide added simultaneously with the activating agents entirely prevented the accumulation of IL-2, pAT 744 (Fig. 4), and pAT 464 mRNA (data not shown), which implied a mechanism of gene induction requiring newly synthesized protein. In contrast, cycloheximide treatment enhanced mRNA levels in the other two groups (Fig. 4 and data not shown).

DISCUSSION

The effects of different signaling pathways with regard to ability to induce a panel of nine novel mitogen-induced genes

are summarized in Table 1. A majority of the genes studied showed some degree of responsiveness to PKC activation alone. However, this signal was insufficient for maximal responsiveness for any of the genes analyzed, since PHA, anti-CD3, and anti-CD2 substantially enhanced the effect of PMA for all of the genes in PB T cells (data not shown), whereas anti-CD28 and ionomycin (Fig. 3 and data not shown) synergized with PMA for a subset of these genes in PB T cells.

The apparent homogeneity of expression patterns for each gene in response to PHA, anti-CD3, or anti-CD2 may reflect the fact that qualitatively, a similar series of intracellular biochemical events results from activating T cells with these agents. This common pattern of gene expression in response to a variety of inducing agents can potentially be mediated at a number of levels in the signaling pathway. At the level of signal generation, either a similar constellation of intracellular messengers is generated or convergence of diverse signaling molecules occurs at a point proximal to their effects on the transcriptional or posttranscriptional regulation of these genes. As another possibility, distinct regulatory motifs controlling these genes may be responsive to divergent signals, with all generating a positive effect on transcription.

Despite the apparent uniformity of PB T-cell responses to stimulation with PHA or anti-CD3 and anti-CD2, regulatory distinctions for induced genes were unmasked by probing the system with ionomycin, which is expected to generate a subset of signals relative to those above (24), and with anti-CD28, which appears to generate a distinct secondary signal(s) (8, 12, 14, 22, 25). Class V genes (Table 1), those that are ionomycin and anti-CD28 nonresponsive, are novel relative to the majority of mitogen-induced genes previously described in T cells (8). Further heterogeneity was observed by analyzing the effects of membrane bypass stimuli in the Jurkat cell line. The fact that five regulatory classes could be discerned among this relatively small group of nine mitogen-induced genes demonstrates the multiplicity of regulatory mechanisms acting at this stage of T-cell mitogenesis.

Although the requirements for gene induction were generally parallel in resting T and Jurkat cells, differences were noted in the regulation of certain genes. Specifically, simultaneous cycloheximide addition abrogated the mitogen induction of pAT 464, pAT 744, and IL-2 in Jurkat cells (Fig. 4 and data not shown) but slightly elevated mRNA levels for these genes in PB T cells (Fig. 2 and data not shown). Secondly, ionomycin synergized with PMA to elevate pAT 237 mRNA levels in PB T cells but not in Jurkat cells, whereas pAT 229 did not respond to ionomycin in PB T cells (data not shown) but did so in Jurkat cells. These disparities may stem from the fact that PB T cells are heterogeneous with regard to population subtypes or from differences in the basal state of activation between quiescent, nontransformed cells and cycling, tumor cells. Alternatively, Jurkat cells may abnormally overexpress or underexpress specific regulatory components required for some gene induction events.

We have demonstrated a striking correlation in the regulation of pAT 464 and pAT 744 with that of IL-2. Indeed, the DNA sequences of pAT 464 and pAT 744 yield a derived amino acid sequence showing a leader peptide and conservation of amino acids at specific positions that are characteristic of one class of lymphokines (P. E. Zipfel, J. Balke, S. G. Irving, K. Kelly, and U. Siebenlist, J. Immunol., in press), and these proteins are secreted into the supernatants of transfected COS cells (P. F. Zipfel, unpublished observations). This regulatory class of genes responds to signals mediated through CD28, in addition to CD2 and CD3 in PB T cells, and exhibits a requirement for two signals in Jurkat cells, and their induction is completely abrogated by cycloheximide treatment in Jurkat cells. A requirement for protein synthesis preceding mRNA induction in Jurkat cells appears to be unique to this class of genes, relative to many other mitogen-induced genes studied here and elsewhere, suggesting a novel and conserved regulatory mechanism for these and most likely other lymphokines. In this regard, it is tempting to speculate that genes having commonality of function will often show coordinate regulation. Elucidation of the function of these gene products and the molecular analysis of their regulation will result from further studies which are now in progress.

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